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PHYLOGENETIC ANALYSIS OF NP AND HN GENE OF NEWLY ISOLATED NDV/Badung-02/AK/14

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ABSTRACT

This study aimed to reveal the phylogenetic characteristic of newly isolated NDV/Badung-02/AK/14 based on NP and HN gene segment. The virus was isolated from suspected Newcastle disease (ND) chicken in backyard farm at Sibang Village, Badung Regency, Bali Province. The isolates were then propagated and confirmed for NDV serologically. RNA isolation was performed by standard Trizol method. Phylogenetic tree analysis of NP gene (nt1020-1561) and HN gene (nt7019-7754) were performed using sequence of Badung-02/AK/14 and selected NDV strains from GenBank. Based on the NP gene sequence, the newly isolate closely related with other NDV strains belong to genotype VII that previously isolated such as Banjarmasin-010/10, Bali-1/07, and Cockatoo/90 with genetic distance 0.2%, 12.6%, and 18.0% respectively. The genetic distance with LaSota/46 virus (genotype II) is 19.6%. Based on HN gene sequence, genetic distance of the Badung-02/AK/14 with other viruses belong to genotype VII such as Banjarmasin-010/10, Bali-1/07, and Cockatoo/90 are 0.4%, 3.7%, and 4.2% respectively. The genetic distance with LaSota/46 virus (genotype II) is 9.0%. There were no difference between the result of nucleotide and amino acid sequence analysis, both in NP and HN gene (P>0.05).

Key words: HN gene, molecular characteristics, NDV, NP gene

ABSTRAK

Tujuan dari penelitian ini adalah untuk monitoring karakteristik filogenetik virus NDV/Badung-02/AK/14 berdasarkan segmen gen NP dan HN. APMV-1 isolat Badung-02/AK/14 telah diisolasi dari kasus Newcastle disease (ND) lapangan di desa Sibang, Kabupaten Badung, Bali. Virus kemudian di propagasi pada telur ayam bertunas (TAB) dan dilakukan uji serologi. Isolasi RNA kemudian dilakukan dengan metode Trizol. Analisis filogenetik kemudian dilakukan pada segmen gen NP (nt 1020-1561) dan HN (nt7018-754). Analisis phylogenetic tree dilakukan untuk membandingkan sekuens gen NP dan HN virus Badung-02/AK/14 dan isolate NDV yang didapatkan dari GenBank. Hasil analisis terhadap segmen gen NP menunjukkan bahwa isolat Badung-02/AK/14 berada satu grup dengan virus pendahulunya dari genotipe VII seperti Banjarmasin-010/10, Bali-1/07, dan Cockatoo/90 dengan jarak genetik masing-masing 0,2%: 12,6%; dan 18,0%. Jarak genetik dengan isolat vaksin LaSota/46 (genotipe II) adalah 19.6%. Selain itu, berdasarkan analisis terhadap segmen gen HN, jarak genetik antara isolat Badung-02/AK/14 dengan isolat lainnya dari genotipe VII seperti yang telah disampaikan adalah masing-masing 0,4%; 3,7%; dan 4,2%. Jarak genetik dengan isolat vaksin LaSota/46 (genotipe II) adalah 9.0%. Tidak terdapat perbedaan hasil analisis sekuens nukleotida dan asam amino baik berdasarkan gen NP maupun gen HN (P>0,05).

Kata kunci: gen HN, karakteristik molekuler, NDV, Gen NP

INTRODUCTION

Newcastle disease (ND) is regarded worldwide as one of the most devastating disease of poultry. This disease is endemic in Indonesia as indicated by the discovery of these cases throughout the year (Kencana *et al*., 2012), and the infection by viscerotropic velogenic strains have been reported (Adi, 2011). The Newcastle disease virus (NDV) is an enveloped virus with negative-sense, single-stranded, nonsegmented RNA genome. The NDV viral genome has 15,186 kb in length which encodes six structural proteins: nucleoprotein (NP), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), RNA polymerase (L), and always in order of 3'-NP-P-M-F-HN-L-5' (Dortmans *et al*., 2011; Hines and Miller, 2012).

The amino acid sequence at the fusion protein (F protein) cleavage site has been shown to be a major determinant of NDV virulence, since the function of its protein in mediating virus penetration into host cells and the formation of syncytium (Adi *et al*., 2009). The NDV will begin to infect the host cell after the attachment to the host cell through a specific receptors called sialic acid receptor, which mediated by the HN protein. The HN protein promotes the attachment and release of the virus. There are two major roles of HN protein, the first one is to mediates recognition, absorption, and penetration of the virus (HA, hemagglutination activity), and the second one acts as an enzyme (NA, neuraminidase avtivity) that removes sialic acid (neuraminic acid) moieties from viral progeny to prevent self-aggregation, favoring the release of virions from the cell (Hines and Miller, 2012). HN protein also plays an important role in tissue tropism (Huang *et al*., 2003). The HN protein between virulent and avirulent virus are different, but it has been confirmed that the pathogenicity of the virus is not influenced by the length of its HN protein, but affects the phylogenetic relationships (Römer-Oberdörfer *et al*., 2003). Research has reported that mutations at the end of the N-glycosylation of HN proteins was reduce the virulence of NDV (Panda *et al*., 2004; Khattar *et al*., 2009).

The NP protein serves as the site for viral RNA synthesis and captures the genomic RNA into the nucleocapsid during replication. NP protein is the first synthesized protein after uncoating process complete. NP protein also has the function to regulate the alteration from transcription of the viral RNA to the synthesis of the genome to form new virions (Fenner *et al*., 2000). Research showed that the NP protein, along with P and L proteins that form the viral replication

complex, have a significant role to the virulence of the NDV (Dortmans *et al*., 2010). Interactions between NP and M proteins involved in the regulation of RNA viral replication, which affect the replication process (Iwasaki *et al*., 2009; Schmitt *et al*., 2010).

Various researches have proved that molecular variation of NDV can occur temporally and spatially, which form various branches on the phylogenetic tree. It has proven could be useful in assessing local and global epidemiology of NDV (Chang *et al*., 2001; Mohamed *et al*., 2009; Brian *et al*., 2012; Diel *et al*., 2012; Parthiban *et al*., 2013). High genetic diversity of NDV is due to high mutation rate of genetic material. The most common cause of mutations in NDV is polymerization error and recombination of nucleic acids (Chare *et al*., 2003; Ramey *et al*., 2013). The high mutation rate of RNA viruses caused by a viral RNA polymerase has no proofreading activity (Martinez *et al*., 2012). The high mutation rate in NDV makes it attractive to continuously being studied. Based on this background, the molecular characteristic of NDV genotypes circulating in the field, should be monitored periodically.

MATERIALS AND METHODS

The NDV/Badung-02/AK/14 was isolated from suspected NDV chicken in backyard farm at Sibang Village, Badung Regency, Bali Province. The isolate were then propagated in embryonated chicken eggs and confirmed for NDV serologically. RNA isolation was performed by standard Trizol method. One Step RT-PCR was performed by mixing 5 µl of R-Mix consisting of buffer, MgCl and dNTP, 0.6 µl of the forward primer, 0.6 µl reverse primer, 2.55 µl double distilled water, 0.25 µl enzyme, and 1 µl RNA template. Two set of primers namely F3s (5'-ATGAAGCAGCTCATGCGTTT-3') and F3r (5'-AGTCGGTGTCGTTATCTTGG-3'), was used to amplify the NP gene (nt1020-1561), and F15s (5'-CAGAGATCACTCACATTCAT-3') and F15r (5'-GCCTAAGGATGTTGACACCT-3') to amplify the HN gene (nt7018-754) (Adi, 2011). The PCR protocol was as follows: 94° C for 45 s, 55° C for 45 s and 72° C for 1 min (adding 5 s per cycle) for 39 cycles, followed by 72° C for 5 min. The PCR products were examined by electrophoresis in 2% (w/v) TAE agarose gel. PCR products of the expected length were purified with a gel extraction kit (GENEAID, Taiwan). The purified PCR products were sequenced in both directions (PT Genetika Science, Jakarta). Phylogenetic analysis of the NDV strain characterized in this study and representative strains from the GenBank was performed using the NP gene (nt1020-1561) and HN gene (nt7018-7754). An unweighed pair group method with arithmetic mean (UPGMA) phylogenetic tree was constructed using MEGA version 4.0 software. Bootstrap probabilities of each node were calculated using 500 replicates. Pairwise distance calculation was performed using compute distance only method with maximum composite likelihood model. Amino acids sequence were analyzed using translated protein sequences tools in aligment explorer of MEGA version 4.0 software.

RESULTS AND DISCUSSION

Nucleoprotein (NP)

Phylogenetic tree analysis of NP gene (nt1020-1561) were performed using sequence of newly isolated Newcastle disease virus Badung-02/AK/14 and sequences of selected NDV strains from GenBank. The results showed that based on the NP gene sequence, the isolate closely related with other NDV strains belong to genotype VII that previously isolated such as Banjarmasin-010/10, Bali-1/07, and Cockatoo/90 with genetic distance 0.2%, 12.6%, and 18.0% respectively. The genetic distance with vaccine strain widely use in Indonesia i.e. LaSota/46 (genotype II) is 19.6% (Figure 1).

NDV/Banjarmasin/010/10 isolate was the isolate that have the highest similarity percentage with the Badung-02/AK/14 isolate (99.9%). This virus was isolated in Banjarmasin in 2010 (Xiao *et al*., 2012). While NDV/Bali-1/07 isolates that have been isolated in Bali in 2007 (Adi *et al*., 2011), only shared 94% similarity. This showed that genetic variation of the NP gene is strongly influenced by the time. The variation rate is more influenced by time (temporal factor) rather than the geographic factors (spatial factor).

This finding is differed from that of the earlier research stated that there was no temporal effect on the genome of NDV (Toyoda *et al*., 1987), which indicates there were no genetic changes as time gone by. This

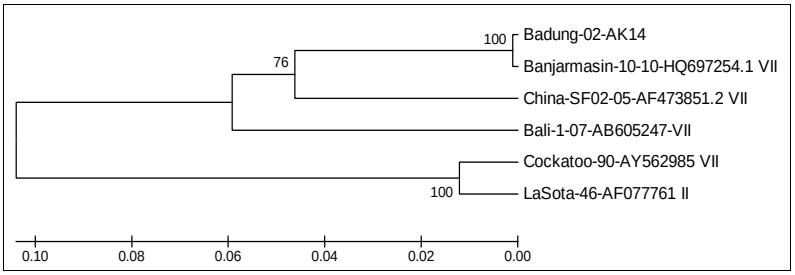


Figure 1. Phylogenetic tree of NDV based on partial sequences of the NP gene from position 1088 to 1560 (473 bps). Phylogenetic analysis performed by bootstrap test with 500 replication methods unweighed pair group method with arithmetic mean (UPGMA) in MEGA4 program. Number at branching point are the bootstrap values in percent

statement is supported by the results of research on homology comparisons of NDV/JP/Ibaraki/SG106/99 isolate compared to other isolates isolated at different times (temporal dimension) has a genome similarity of 99-100% (Umali *et al*., 2013). Another study conducted by Garcia *et al*. (2013) also showed the temporal factor, along with the spatial (geographic) factor has no effect on the NDV genome.

Phylogenetic analysis showed that based on NP genes segment analysis, NDV/Badung-02/AK/14 isolate was in the same cluster with the other genotype VII isolates such as Banjarmasin-1, Bali-1/07, China (NA-1), China (NA-1M), and Goose-China-SF02 (Figure 1). To determine the genotype of the NDV, the fragment of F gene are generally being used. But in this study, Badung-02/AK/14 was found belong to genotype VII. It showed that there was no recombination found along the NP gene fragment (nt1020-1561). It is supported by previous research on F gene sequence of Badung-02/AK/14 which found that this isolate was genotype VII (Putra *et al.*, 2016).

Recombination of NDV genome is commonly found on the NP gene. Research conducted by Cho *et al*. (2008) revealed a recombination on a segment of NP gene between NDV/Cocckato/90 isolates and Lasota strain which belongs to genotype II (Figure1). Analysis of amino acid alignment showed that there was no variation on the first 23 amino acids (sequence number 363-385) of NDV/Badung-02/AK/14 isolate compared with other genotype VII isolates such as Bali-1/07, China(NA-1), China(NA-1M), Goose-China-SF0; and vaccine isolates Lasota (genotype II). Variations began to appear at the 24th amino acid (sequence no. 386). The NDV/Badung-02/AK/14 isolates encoding valine, while the other isolates encoding glycine (Figure 2).

The result of analysis on 157 amino acids of NP protein showed that NDV/Badung-02/AK/14 isolate was closely related with other NDV isolates such as Bali-1/07, China (NA-1), China (NA-1M), Goose-China-SF0, and Lasota with the similarity percentages of 92.2%, 96.7%, 96.7%, 96.7%, and 86.6% respectively (Figure 2). Remarkable interesting result is the high amino acid sequence variation between NDV/Badung-02/AK/14 and Lasota, with genetic distance of 13.4%. In order to confirm the analysis result of nucleotide sequence and amino acid sequence, independent sample T-test was done to compare the similarity percentages. The results of this test showed that based on NP gene segment sequence, there were no difference between the result of nucleotide and amino acid sequence analysis (P>0.05).

Hemagglutinin-Neuraminidase (HN)

Based on HN gene sequence (nt7019-7754), genetic distance of the Badung-02/AK/14 with other viruses belong to genotype VII such as Banjarmasin-010/10, Bali-1/07, and Cockatoo/90 with genetic distance 0.4%, 3.7%, and 4.2% respectively. The genetic distance with LaSota/46 virus (genotype II) is 9.0% (Figure 3).

Although the complete genome sequence analysis of the HN gene among NDV/Banjarmasin/010/10, Gianyar/013/10, Kudus/018/10, and Sragen/014/10 showed an average similarity of 99.9% (Xiao *et al*., 2012), but the analysis of gene segments on position 7021-7506 of the whole NDV genome, has the similarity of 100%. All of those strains were isolated at the same years (2010), with a high geographical variation (Banjarmasin, Gianyar, Kudus, and Sragen). This indicates that the genetic variation of NDV was not influence by geographical factors (spatial). But over the time from 2010 to 2014, the genetic distance became wider from 0.10% to 0.80%. It showed that the temporal factors have an impact on genetic variation in the HN gene.

From the phylogenetic analysis results (Figure 3), HN genes from NDV/Badung-02/AK/14 isolate was in the same cluster other genotype VII isolates such as Banjarmasin/010/10, Gianyar/013/10, Kudus/018/10, and Sragen/014/10. It can be concluded that there were no genetic recombination along the HN gene fragment (nt7019-7754).

The results of analysis on 165 amino acids of HN protein showed that NDV/Badung-02/AK/14 isolates was closely related with other NDV isolates such as Bali-1/07, Gianyar/013/10, Kudus/018/10, Banjarmasin/010/10, and Lasota with the similarity percentages of 94.5%, 100%, 100%, 100%, and 91.5%, respectively (Figure 4). There was a difference result between nucleotide and amino acids sequence. The nucleotide sequence analysis revealed a genetic

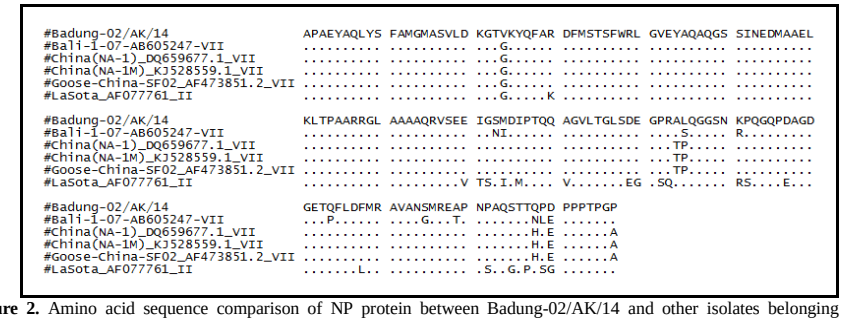


Figure 2. Amino acid sequence comparison of NP protein between Badung-02/AK/14 and other isolates belonging to the genotype VII, and one vaccine isolate Lasota. This analysis indicates the amino acid sequence 363-519 of the whole amino acid of NDV

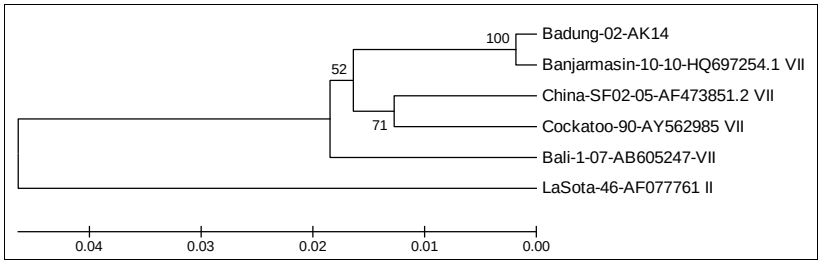
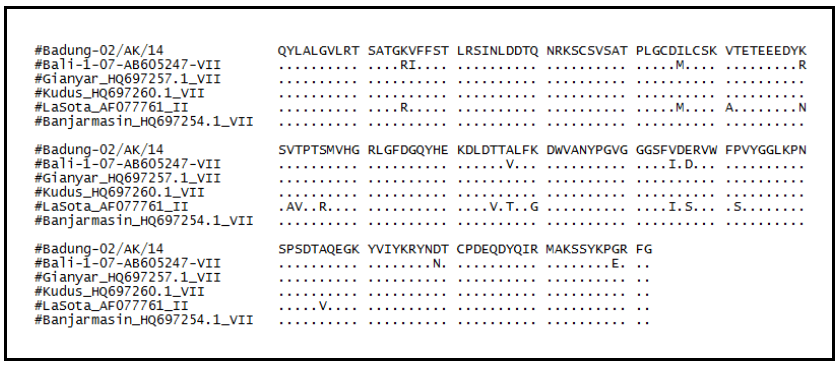


Figure 3. Phylogenetic tree of NDV based on partial sequences of the HN gene from position 7021 to 7506 (486 bps). Phylogenetic analysis performed by bootstrap test with 500 replication methods unweighed pair group method with arithmetic mean (UPGMA) in MEGA4 program. Number at branching point are the bootstrap values in percent



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#Badung-02/AK/14              QYLALGVLRT SATGKVFFST LRSINLDDTQ NRKSCSVSAT PLGCDILCSK VTETEEEDYK
#Bali-1-07-AB605247-VII       .......... ....RI.... .......... .......... .....M.... .........R
#Gianyar_HQ697257.1_VII       .......... .......... .......... .......... .......... ..........
#Kudus_HQ697260.1_VII         .......... .......... .......... .......... .......... ..........
#LaSota_AF077761_II           .......... ....R..... .......... .......... .....M.... A........N
#Banjarmasin_HQ697254.1_VII   .......... .......... .......... .......... .......... ..........

#Badung-02/AK/14              SVTPTSMVHG RLGFDGQYHE KDLDTTALFK DWVANYPGVG GGSFVDERVW FPVYGGLKPN
#Bali-1-07-AB605247-VII       .......... .......... ......V... .......... ....I.D... ..........
#Gianyar_HQ697257.1_VII       .......... .......... .......... .......... .......... ..........
#Kudus_HQ697260.1_VII         .......... .......... .......... .......... .......... ..........
#LaSota_AF077761_II           .AV..R.... .......... ....V.T..G .......... ....I.S... .S........
#Banjarmasin_HQ697254.1_VII   .......... .......... .......... .......... .......... ..........

#Badung-02/AK/14              SPSDTAQEGK YVIYKRYNDT CPDEQDYQIR MAKSSYKPGR FG
#Bali-1-07-AB605247-VII       .......... ........N. .......... ........E. ..
#Gianyar_HQ697257.1_VII       .......... .......... .......... .......... ..
#Kudus_HQ697260.1_VII         .......... .......... .......... .......... ..
#LaSota_AF077761_II           .....V.... .......... .......... .......... ..
#Banjarmasin_HQ697254.1_VII   .......... .......... .......... .......... ..
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Figure 4. Amino acid sequence comparison of HN protein between Badung-02/AK/14 and other isolates belonging to the genotype VII, and one vaccine isolate Lasota. This analysis indicates the amino acid sequence 2341-2502 of the whole amino acid of NDV

distance of 0.8% between NDV/Badung-02/AK/14 and other isolates (Gianyar/013/10, Kudus/018/10, Banjarmasin/010/10), when the amino acid sequence analysis showed there were no genetic distance (shared similarity of 100%) among this isolates. This indicates that there were a silent mutation in the HN gene segments. In order to confirm the analysis results of nucleotide sequence and amino acid sequence, independent sample T-test was done to compare the similarity percentages. The result of this test showed that based on NP gene segment sequence, there were no difference between the result of nucleotide and amino acid sequence analysis (P>0.05).

CONCLUSION

NDV isolate Badung-02/AK/14 is belong to genotype VII based on ND gene and HN gene analysis. And the genetic distance is very close with previously isolated NDV Banjarmasin-010/10. It seems that the genetic distance is strongly influenced by temporal factor.

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